

Identification of lactic acid bacteria in sayur asin from Central Java (Indonesia) based on 16S rDNA sequence

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Abstract

Sayur asin is commonly known in Indonesia, in particular Central Java, as a spontaneous fermented mustard product by epiphytic lactic acid bacteria (LAB) from the plants. However, information regarding LAB diversity in Indonesia is lacking and fragmentary. Therefore, efforts on inventory of LAB from sayur asin in Indonesia has been conducted since April 2013 in order to determine its diversity. In the current study, LAB diversity in five samples of sayur asin collected from Solo, Yogyakarta, and Semarang (Central Java) is reported. LAB were isolated from both of fermented mustards and fermenting liquors. Molecular identification of the isolates was conducted based on 16S rDNA sequence data. A total of 246 isolates of LAB was successfully isolated and identified. The bacteria belong to 11 species, viz, *Lactobacillus farciminis* (15 isolates), *L. fermentum* (83 isolates), *L. namurensis* (18 isolates), *L. plantarum* (107 isolates), *L. helveticus* (1 isolate), *L. brevis* (1 isolate), *L. versmoldensis* (3 isolates), *L. casei* (12 isolates), *L. rhamnosus* (2 isolates), *L. fabifermentans* (3 isolates) and *L. satsumensis* (1 isolate). The current study revealed that *L. plantarum* and *L. fermentum* as common LAB used in sayur asin production from Central Java, Indonesia.

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Introduction

Sayur asin has been known as traditional fermented mustard produced and consumed in many areas in Indonesia for years (Puspito and Graham, 1985). Manufacture of fermented mustard was made by addition of the salt to vegetables, allowing the growth of certain fermentable microorganisms, resulting in sensory changes bearing acidic and unique characteristics to the mustard (Chiou, 2004). Epiphytic lactic acid bacteria (LAB) of which initially amounted to slightly between 10-1000 cfu/g plant (0.001 to 1% of the total population of microorganisms) became dominant within the microorganism population in in the fermented mustard as a result of the addition on the anaerobic treatment and salt on the vegetables (Daeschel *et al.*, 1987; Azcarate and Todd, 2010).

According to Chao *et al.* (2009), fermented mustard is a good source for the exploration of LAB. Differences in the manufacture of fermented mustard, salt concentration, temperature during the fermentation process, and the level of anaerobiosis affect the selection of certain LAB species of in the

mustard fermentation. Puspito and Graham (1985) reported that there was a difference in manufacturing techniques in the mustard fermentation in Indonesia. Several locations in central Java, such as Solo, Yogyakarta and Semarang, have traditionally been known as producers of sayur asin in Indonesia. Therefore, exploration of LAB in these areas will be important to reveal LAB diversity in Indonesian traditional fermented mustard.

In the development of LAB taxonomy, several species of LAB have been recognized as complex species, which is difficult to distinguish by conventional techniques, such as physiological and biochemical tests. Therefore, increasing the numbers of LAB species which have phenotypic and physiological similarities using the conventional techniques often resulting in the misidentification (Vogel and Matthias, 2008; Nikolova *et al.*, 2009). In addition, the conventional techniques are also time-consuming, not reproducible, and do not give accurate results (Yu *et al.*, 2009). Development of molecular methods based on nucleotide sequence data from ribosomal DNA has complemented

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identification methods in food microbiology. Molecular identification method is more accurate and sensitive, rapid, reproducible, does not influenced by environmental factors, and more reliable (Rosetti and Giorgio, 2005). The molecular method is not only useful for differentiation and taxonomy, but also for knowing evolution of bacterial involved in the process of foods fermentation (Rosetti and Giorgio, 2005).

In Indonesia, however, application of molecular technique in identification of LAB from local fermented mustard such as sayur asin, is still lacking and fragmented due to limited knowledge and its expensive cost. Majority of previous studies on LAB diversity in Indonesia was mainly conducted based on biochemical and physiological analyses (Puspito and Graham, 1985). Therefore, it is necessary to carry out inventory and exploration study of LAB in Indonesia based on molecular technique. It is expected that the results from this study will revise several LAB names previously published in sayur asin from Indonesia, because the molecular technique provides a more accurate identification than those of conventional methods.

Materials and Methods

Sampling

Five samples (contain fermenting liquor and fermented mustard) were collected from traditional manufactures in three areas in central Java (Solo, Semarang, and Yogyakarta) (Table 1). In detail, two samples (SL1 and SL2) were collected from Solo, two samples (YK1 and YK2) were collected from Yogyakarta, and one sample (SM1) was collected from Semarang. The samples were taken aseptically and packaged into a plastic bag, then stored at 4°C in ice box. Analyses and isolation were immediately performed in laboratory the day after sampling.

pH measurement

The pH values of fermenting liquor were determined by a pH meter HM-25R (TOA OKK).

Enumeration of LAB

Enumeration of LAB was conducted by spread plate method (Chao *et al.*, 2009). Amount of 1 ml fermenting liquor sample mixed with 0.85% (w/v) NaCl solution. Dilution of the mixed solution (10^{-3} to 10^{-6}) were spread directly onto the surface of MRS (de Man, Rogosa, and Sharpe)-agar containing NaCl 1%. To distinguish acid-producing bacteria from other bacteria, 1% CaCO_3 was added to the MRS-agar plates (Chen *et al.*, 2010). Samples were incubated

Table 1. Sampling location, sampling date, origin of mustard, fermentation time, pH value and cell counts of lactic acid bacteria

Sample code	SL1	SL2	YK1	YK2	SM1
Sampling Location	Pasar Gede Solo Hardjonagoro Jl. Urip Sumohardjo, Sudiroprajan Jebres, Solo		Pasar Pathuk Yogyakarta Jl. Bhayangkara, Yogyakarta		Jl. Tregguli Gg. 2, No 16. Kelurahan Karang Kidul, Kota Semarang
Sampling date	April, 12, 2013	April, 12, 2013	April, 13, 2013	April, 13, 2013	August, 22, 2013
Origin of mustard	Bandung City, of West Java, Indonesia				
Fermentation time (day)	3	2	3	3	4
pH of fermenting liquor	3.38	3.44	3.38	3.40	3.42
TPC of fermenting liquor (cfu/ml)	60×10^6	47×10^6	247×10^6	191×10^6	181×10^6

under anaerobic condition in anaerobic jar at 28°C for 4 days. The colony surrounded by a clear zone are counted.

Isolation of LAB

Amount of 1 g fermented mustard was crushed and mixed with 0.85% (w/v) NaCl solution, and about 1 ml of fermenting liquor mixed with 0.85% (w/v) NaCl solution separately diluted serially (10^{-3} to 10^{-6}). A serial dilution was spread directly onto the surface of MRS agar 1% CaCO_3 plates containing NaCl 1% and NaCl 6% (Chen *et al.*, 2006). Samples were incubated under anaerobic condition in anaerobic jar at 28°C for 4 days. Colonies of acid-producing bacteria identified by a clear zone surrounded each colony, and the colony with distinct morphologies (e.g., in terms of colour, shape and size) were further purified by streaking at least twice on MRS-agar containing NaCl 1% (Chao *et al.*, 2009). Only Gram-positive, catalase-negative strains were selected (Tiwari *et al.*, 2003). The selected strains were stored at -80°C in MRS broth containing 10% glycerol (de Valdez, 2001).

DNA extraction, PCR amplification and sequencing

Genomic DNA from each LAB strain was isolated by a guanidinium thiocyanate protocol as described by Pitcher *et al.* (1989). Amplification of the 16S rDNA was carried out using primer pairs of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Patil *et al.*, 2010). The amplification was performed in a final volume of 50 µl. Each reaction mixture contained 50 µl volume containing 25 µl of Go Taq® Green Master Mix 2x (Promega), 4 µl of 10 primer 27F (First Base, Malaysia) and, 4 µl of 10 µM primer 1492R (First Base, Malaysia) and 16 µl of nuclease free water (NFW) (Nacalai) and 1 µl of 10-100 ng DNA template. A negative-DNA control was performed by adding 1 µl of NFW. PCR was carried out in Eppendorf thermal-cycler (Eppendorf, Germany). The amplification program was set as follows: 94°C for 1.5 min; 30 cycles of 95°C for 30 s, 50°C for 30

s, 72°C for 1.5 min; and 72°C for 5 min. The PCR products were analyzed by electrophoresis using 1.0% (w/v) agarose gel in 1X Tris-acetate-EDTA (TAE) buffer (Promega) at 100 V for 20 min and stained by ethidium bromide. A 1 kb Plus DNA ladder (Invitrogen) was used as size standard. The gels were visualized using UV transilluminator (Atta). The PCR products were sent to Macrogen Laboratories (South Korea) for sequencing. Single-pass sequencing was performed on each template using primer 27F. To confirm the identity of certain isolates with homology below 99%, full-length sequencing of 16S rDNA was performed with universal primers: 27F, 518F, 800R, and 1492R.

Phylogenetic analyses

Sequence homologies were examined by comparing the sequences obtained with 16S rDNA sequences available in the nucleotide databases of the GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>), using the Basic Local Alignment Search Tool (BLAST) program. Multiple sequence alignment of 42 new sequence of LAB from sayur asin with 11 sequences of LAB type strains was conducted by Clustal X software version 2.0.11 (Thompson et al., 1997). *Escherichia coli* strain JCM1649T (X80725) was used as outgroup. Phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) implemented in Clustal X. The stability of the tree was assessed by bootstrap method (Efron, 1979) using 1000 replications. New sequences used in the phylogenetic analyses were submitted to DDBJ GenBank database under accession number AB889690 - AB889731.

Results

Determination of pH and enumeration of lactic acid bacteria

A pH value of the fermenting liquor from the total five samples range from 3.38 to 3.44. Average of total plate count (TPC) of LAB in the five samples is $47 - 247 \times 10^6$ cfu/ml. Sample of sayur asin from Yogyakarta (YK1) showed the highest LAB population, while sample from Solo (SL1) contains the lowest number of LAB population (Table 1).

Identification of LAB based on phylogenetic analyses of 16S rDNA sequences

A total 246 LAB isolates were subjected to 16S rDNA sequence analyses. Of them, only 42 new LAB sequences were included in the phylogenetic analyses. The remaining 204 new LAB sequences were excluded from the phylogenetic analyses

because of having identical DNA sequence with the 42 LAB sequences (data not shown). Full-length 16S rDNA sequence analysis was performed to the strains Y1_24D, Y2_07D, and Y2_26D. The phylogenetic tree generated from NJ analyses showed that LAB from sayur asin divided into 11 independent clades, viz, *L. plantarum* (Orla-Jensen) Bergey et al. (1934) (1923) (93.7% BS), *L. fabifermentans* De Bruyne et al. (2009) (99.6% BS), *L. versmoldensis* Kröckel et al. (2003) (61.6% BS), *L. farciminis* Reuter (1983) (83.4% BS), *L. brevis* (Orla-Jensen) Bergey et al. (72.3% BS), *L. namurensis* Scheirlinck et al. (2007) (99.6% BS), *L. satsumensis* Endo and Okada (2005) (100% BS), *L. rhamnosus* (Hansen) Collins et al. (1989) (67.3% BS), *L. casei* (Orla-Jensen) Hansen and Lessel (1971) (70% BS), *L. fermentum* Beijerinck (1901) (99.9% BS) and *L. helveticus* (Orla-Jensen) Bergey et al. (1925) (100% BS). These independent monophyletic clades clearly determined the species name of Indonesian LAB in sayur asin collected in Central Java.

Lactic acid bacteria (LAB) composition in five samples of sayur asin

The highest diversity of LAB was found on sample SL2 and YK1 with seven and six LAB species found in each sample, respectively (Table 2). *Lactobacillus plantarum* and *L. fermentum* were found as common and dominant LAB used in sayur asin in Solo, Semarang and Yogyakarta. *Lactobacillus helveticus*, *L. brevis*, *L. versmoldensis*, *L. rhamnosus*, *L. fabifermentans* and *L. satsumensis* were rarely found in the samples.

Discussion

The microbiology of mustard fermentation is similar to other fermented vegetables, because the microorganisms responsible for the fermentation process are mainly belonging to LAB (Puspito and Graham, 1987; Chiou, 2004). Activities of LAB during the fermentation have been known decreasing pH of the fermented foods. The final pH resulted from the fermentation process is possibly related to incubation time. Our data showed SL1 (pH = 3.38) and SL2 (pH = 3.44) which were collected from the same location (Solo), having different fermentation time (Table 1). However, this condition is not always consistent due to the samples from Yogyakarta did not show the same result. The change in acidity (pH) of sayur asin by the LAB can be considered as main indicators of a good fermentation (Bomrungnok et al., 2007).

The total cell count in five samples was vary

Table 2. Lactic acid bacteria (LAB) composition in five samples of fermented mustard

No.	Species	SL1	SL2	YK1	YK2	SM1	No of isolate
1	<i>L. farciminis</i>	(2)	(1)	(6)	(6)	-	15
2	<i>L. fermentum</i>	(17)	(11)	(27)	(25)	(3)	83
3	<i>L. namurensis</i>	(8)	(6)	(4)	-	-	18
4	<i>L. plantarum</i>	(3)	(36)	(20)	(10)	(38)	107
5	<i>L. helveticus</i>	-	(1)	-	-	-	1
6	<i>L. brevis</i>	-	-	(1)	-	-	1
7	<i>L. versmoldensis</i>	-	-	(3)	-	-	3
8	<i>L. casei</i>	-	(1)	-	(1)	(10)	12
9	<i>L. rhamnosus</i>	-	(2)	-	-	-	2
10	<i>L. fabifermentans</i>	-	-	-	-	(3)	3
11	<i>L. satsumensis</i>	-	-	-	-	(1)	1
Total of isolates		30	58	61	42	55	246

between 47 - 247 x 10⁶ cfu/ml. According to Chao *et al.* (2009), the variety of LAB population in the fermented mustard was influenced by different fermentation process and treatments, especially the salt concentration. During the process, the squeezed and salt treatment enhanced the water and nutrient were drawn out of the vegetable tissues by high osmotic pressure (Chiou, 2004). Beside that, the varying conditions of anaerobiosis, moisture levels, and temperature resulted in changes in the population balance and selected for spontaneous fermentation by lactic acid bacteria (Azcarate and Todd, 2010). The growth of the lactic acid bacteria is also influenced by nutrient movement from plant material into the surrounding liquid (Daeschel *et al.*, 1984). However, we could not determine the relationship between population of LAB and these factors in different sayur asin samples, in particular amount of salt concentration, because of lacking standard amount of salt used in the mustard fermentation from Solo, Yogyakarta, and Semarang.

The identification based on phylogenetic analyses of a total 246 sequences generated from 16S rDNA region showed that these LAB belonging to a single genus, *Lactobacillus*. It is not surprising since members of *Lactobacillus* have commonly been found and used as agents of various fermentation foods and drinks. The genus *Lactobacillus* has been known as the largest genera of lactic acid bacteria with over 145 recognized species (Euzéby, 1997). The members of *Lactobacillus* are also very heterogenous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. Many species have been found in many spontaneous lactic fermentation such as vegetable and silage fermentations (Axelsson, 2004).

The eleven species found in this study - *L. farciminis*, *L. fermentum*, *L. namurensis*, *L. plantarum*, *L. helveticus*, *L. brevis*, *L. versmoldensis*, *L. casei*, *L. rhamnosus*, *L. fabifermentans* and *L. satsumensis* - have also been reported found in different fermented foods (Ludwig *et al.*, 2009). These species have been characterized having different fermentation type

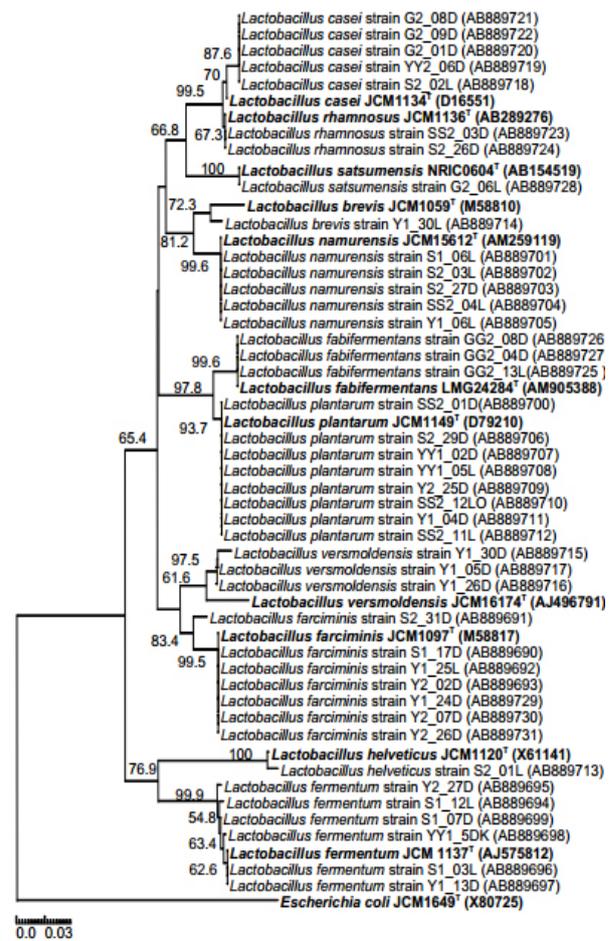


Figure 1. Phylogenetic tree based on 16S rDNA sequence analysis of representative isolates from five sample fermented mustard. The tree was constructed by the neighbor-joining method, and *Escherichia coli* was used as the outgroup. Bootstrap values expressed as of 1000 replication and scale bar represent 0.03 sequence divergence.

such as obligately homofermentative, facultatively heterofermentative, obligately heterofermentative (Felis and Franco, 2007), and have been reported no pathogenic to human or animal (Azcarate and Tod, 2010). Therefore, the five products of sayur asin from Solo, Yogyakarta, and Semarang contains LAB which are safe to human for consumption.

These results revise the previous data reported by Puspito and Graham (1985). Several species which were not found by Puspito and Graham (1985), have been determined in the current study. These include *L. namurensis*, *L. versmoldensis*, *L. rhamnosus* (previously known as *L. casei* subsp. *rhamnosus*), *L. fabifermentans* and *L. satsumensis*. The result showed each sample consist of varying species. In the five samples of fermented mustard showed *L. plantarum* and *L. fermentum* were predominant. It was probably due to *L. fermentum* and *L. plantarum* are more acid tolerant, and often dominate fermentation processes of vegetables and cereals, in particular because of

their ability to transport and metabolize different carbohydrates (Weckx *et al.*, 2010). Lactic acid bacteria either *L. plantarum* and *L. fermentum* likely play important roles in the mustard fermentation.

The phylogenetic tree showed a close relationship among particular LAB species (Figure 1). A clade containing *L. plantarum* isolates found sister clade to *L. fabifermentans* (97.8% BS). This data is in concordance with De Bruyne *et al.* (2009) who reported that *L. fabifermentans* belonging to the *L. plantarum* species complex. This complex includes *L. plantarum*, *L. fabifermentans*, *L. paraplantarum* Curk *et al.* (1996) and *L. pentosus* Zanoni *et al.* (1987). A close phylogenetic relationship was also shown between *L. rhamnosus* and *L. casei* (99.5% BS). In fact, *L. rhamnosus* (= *L. casei* subsp. *rhamnosus*) was emerged as independent species based on DNA-DNA hybridization analyses (Collins *et al.*, 1989). Our phylogenetic analyses based on 16S rDNA sequences apparently supported that *L. rhamnosus* belong to *L. casei* species complex. Claesson *et al.* (2008) noted that due to many species complexes exist in LAB, therefore, extensive polyphasic approach which is combining conventional and phylogenomic methods are necessary to carry out in resolving the systematic of LAB.

Conclusions

Eleven species of LAB, viz, *L. farciminis*, *L. fermentum*, *L. namurensis*, *L. plantarum*, *L. helveticus*, *L. brevis*, *L. versmoldensis*, *L. casei*, *L. rhamnosus*, *L. fabifermentans* and *L. satsumensis*, were determined in this study. Among them, *L. plantarum* and *L. fermentum* were found as common LAB used in the fermented mustard products from Solo, Semarang, and Yogyakarta of Central Java, Indonesia.

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